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WO 03/095479 A1

(54) Title: MULTIPLE BRANCH PEPTIDE CONSTRUCTIONS COMPRISING FROM 2 TO 16 BRANCHES OF THE PEPTIDE RQGYS OR FROM 8 TO 16 BRANCHES OF THE PEPTIDE RQGY PROVIDING FROM THE HIV GP41 GLYCOPROTEIN

(57) Abstract: Multiple branch peptide constructions formed from peptides derived from the envelope transmembrane glycoprotein gp41 of HIV, and including from 2 to 16 branches of the peptide RQGYS or from 8 to 16 branches of the peptide RQGY, show increased receptor affinity and prevent cell-to-cell fusion. They have a direct virostatic effect. These MBPCs are able to neutralize in vitro the different steps of virus envelope/cell membrane fusion, and infected cell membrane/uninfected cell membrane fusion of several strains of HIV-1 and HIV-2. These results open a potential use in treatment of HIV infection.

MULTIPLE BRANCH PEPTIDE CONSTRUCTIONS COMPRISING FROM 2 TO 16 BRANCHES OF THE PEPTIDE RQGYS OR FROM 8 TO 16 BRANCHES OF THE PEPTIDE RQGY PROVIDING FROM THE HIV GP41 GLYCOPROTEIN

The invention relates to multiple branch peptide constructions (MBPCs) and to their use in the treatment of Human Immunodeficiency Virus (HIV) infections.

The use of radially branched systems in polymers has been known for a long time in classical polymer chemistry. This system has been used by J.P.Tam [Proc. Natl. Acad. Sci. USA 85, 5409-5413 (1988)] to develop antigens without the use of ambiguous carriers, using lysine skeletons. Those antigens were designed to generate vaccines against a variety of diseases. Antigens for generating vaccines against HIV infection are described by Tam in WO93/03766. He called them Multiple Antigenic Peptide Systems (MAPS), consistent with their conceived use.

The present inventors, along with others, found that similar constructions with shorter peptides derived from the V3 loop of the surface envelope glycoprotein gp120 of HIV offered a direct therapeutic approach to the treatment of HIV infections, as described in WO95/07929. The name MAPS was then inappropriate, and the compounds were renamed as MBPCs. The MBPCs of WO95/07929 interfered with the virus envelope – cell membrane fusion step and also the infected cell membrane – uninfected cell membrane fusion step, either step being thought to be indispensable for cell infection, virus multiplication and the spread of virus in the host organism, by blockading the CD4 receptor present in cells such as lymphocytes and macrophages, apparently by attaching to a membrane co-receptor which is distinct from the CD4 binding receptor, without causing the cell to lose its ability to be activated by other antigens or mitogens.

Subsequently the inventors discovered further MBPCs which are effective as treatments for HIV infections. These further MBPCs incorporated peptides derived from the HIV envelope transmembrane glycoprotein gp41, and were described in WO 98/29443. They comprised a core matrix to which are bonded from 2 to 16, and preferably from 4 to 16 peptides, each of which comprises the sequence RQGY

preceded by from 0 to 4 amino acid residues and succeeded by from 2 to 4 amino acid residues, but not including more than 10 amino acids. The preferred MBPC in WO 98/29443 had 8 branches of the peptide RQGYSPL on a lysine core matrix. Subsequently to the invention described in WO 98/29443, it was been established that the MBPC $[RQGYSPL]_2$-K-βA is effective but that the MBPC $[RQGYSP]_2$-K-βA is less so. This was thought to confirm the lower limit of 6 amino acids in the peptide branches of the MBPCs.

It has now most surprisingly been found that certain short MBPCs retain the effectiveness of the compounds of WO 98/29443. Accordingly, the invention provides a multiple branch peptide construction comprising a core matrix to which are bonded from 2 to 16 peptides, each of which comprises the amino acid sequence RQGYS. The invention further provides a multiple branch peptide construction comprising a core matrix to which are bonded 8 or 16 peptides, each of which comprises the amino acid sequence RQGY. In jurisdictions in which methods for treatment of the human or animal body by therapy are patentable, the invention also provides a method for the therapeutic treatment of patients with HIV infections, the method comprising administering such an MBPC to the patient, preferably in such an amount as to induce in the patient a blood concentration of the MBPC of from 10^{-7} to 10^{-4} molar.

The core matrix is a dendritic polymer which is branched in nature, preferably with each of the branches thereof being identical. The core matrix is based on a core molecule which has at least two functional groups to which molecular branches having terminal functional groups are covalently bonded. Suitable core molecules include ammonia or ethylenediamine. Suitable molecular branches include acrylic ester monomers which are polymerized onto the core molecule. Such molecules may be created to present varying number of branches, depending on the number of monomers branched from the core molecule. The preferred core molecule is lysine. A central lysine residue is bonded to two lysine residues, each through its carboxyl group, to one of the amino groups of the central lysine residue. This provides a molecule with four amino groups, which may be the core matrix for an MBPC having four peptides. Alternatively, one can provide a molecule with eight branches by bonding four lysine residues through their carboxyl groups to one of the amino groups

of the lysine residues which are attached to the central lysine. This molecule can serve as the core matrix for an MBPC having eight peptides or can alternatively receive eight lysine residues to form a core matrix for an MBPC having sixteen peptides.

The C-ends of peptides are covalently bonded to each of the branches of the core matrix to form the MBPC. The resulting molecule has a cluster of peptides at the surface and an interior core matrix which is not presented and is therefore not antigenic.

Spacers may, if desired, be included between the peptides and the core matrix. The carboxyl group of the first lysine residue may be left free, amidated, or coupled to β-alanine or another blocking compound.

Peptides can include D or L-amino acid residues. D amino acids last longer in vivo because they are harder for peptidase to cut, but the L amino acids have better activity, as discussed below.

Moreover, peptide analogues, synthetic constructs using the carbon skeleton of peptides but omitting the -CONH- peptide bonds, can be employed in place of peptides. Thus, it should be understood that references to peptides herein may also be taken to include peptide analogues. It is believed that peptide analogues will be more resistant to peptidase and last longer in vivo.

The preferred MBPCs for use in this invention are

Short RL: $[RQGYS]_2$-K-βA-OH and

RL3: $[RQGY]_8$-$(K)_4$-$(K)_2$-K- βA-OH

The OH terminal shown above on the β-alanine indicates the carboxyl group thereof, with the amino group being attached to the carboxyl group of the lysine residue. The carboxyl group of the β-alanine may alternatively be modified to form a carboxamide terminal.

Short RL has 2 peptides of 5 amino acids each as compared to 8 peptides of 7 amino acids each in $[RQGYSPL]_8$-$(K)_4$-$(K)_2$-K-βA-OH (RL1, the preferred compound of

WO 98/29443). Short RL will therefore be much cheaper to make. As it has an efficacy similar to that of RL1, it represents a significant advance.

WO 99/34777 describes the benefits of liposomes which have a sufficient size for white blood cell internalisation and contain MBPCs useful for the treatment of HIV. Such liposomes may have an average size of greater that 150 nm, and preferably of from 250 nm to 400 nm. They desirably contain above 8% by weight of the MBPC. They make the encapsulated MBPCs more available to the lymphatic system and to the lymphocytes and macrophages which are the target cells for HIV and therefore for anti-HIV substances. It will be understood that the MBPCs of this invention may be contained in such liposomes.

The preparation of the MBPCs of the invention, having a branched core with peptides attached thereto, can be effected by methods known in the art, see e.g. Tam et al, J. Immun. 148, 914-920 (1992). Preferably, for small quantities (under one kilogram), a solid phase method is used to obtain the MBPCs. Stepwise assembly of the peptide chains can be carried out automatically on 4-(oxymethyl)-phenylacetamidomethyl copoly(styrene-1% divinyl benzene). The Boc/benzyl strategy may be used, including a systematic double coupling scheme with hydroxybenzotriazole active esters (Boc-amino-acid-OBt). The final cleaving from resin is effected with strong acid, such as anhydrous hydrogen fluoride (1 hour at 0øC). The MBPC is then washed withdiethyl ether and solubilized in water. After lyophilization, the MBPC may be pre-purified on a P2 or G15 type molecular filtration column, equilibrated with 0.1N acetic acid. The eluate fraction may then be recovered. The purification step is achieved by using C8 or C18 reversed-phase HPLC. The MBPC may be characterized by its amino acid content after acid hydrolysis (6N HCl, 115°C, 24 hours) and electrospray mass spectrometry.

EXPERIMENTAL SECTION

Materials

N-α-fluorenylmethyloxycarbonyl (Fmoc) amino acid derivatives were purchased from Perkin-Elmer. All solvents were analytical-grade commercial products from

Perkin-Elmer or SDS (Peypin, France).

Human PBLs obtained from healthy HIV-seronegative donor (Etablissement Français du Sang, Marseille, France) were isolated by ficoll-Hypaque gradient centrifugation. Cells were cultured in R10 medium supplemented with 20 units/ml of interleukin-2 (IL-2, Proleukin, Chiron, The Netherlands). R10 medium consists of RPMI 1640 supplemented with 2 mM ultraglutamine (BioWhittaker, Verviers, Belgium), penicillin (100 units/ml), streptomycin (100 µg/mi), and 10 % heat-inactivated fetal calf serum (BioWhittaker). Cells were first stimulated with phytohemagglutinin (20 µg/ml)-supplemented R10 (PHA P, DIFCO, Detroit, MI, USA) for three days. Then, the medium was replaced with R10 supplemented with IL2 (20 units/ml), and subsequent cultures and experiments were carried out in this medium in a 37°C humidified incubator with 5% CO_2.

Viral stocks of the TCLA X4 HIV-$1_{NL\ 4-3}$ (obtained from I. Hirsh, INSERM U 372, Marseille, France) (Adachi et al., 1986; Barre-Sinoussi et al., 1983) were produced in permissive CEM cells. HIV-1_{Hx10} and HIV-1_{MN} (obtained from Q. Sattentau) were propagated in H9 cells. Cultured supernatants from infected cells were collected at the peak of maximal viral production as assessed by p24 assay, and residual cells were removed by centrifugation at 4°C (2,000 rpm/5 min). They were sampled and stored at -80°C. The viral stock infectious titer (50 % tissue culture infectious dose, $TCID_{50}$) was established on C8166 cells and PBL.

Chemical synthesis and physicochemical characterisation of synthetic MBPCs

Stepwise elongation of MBPCs was carried out on 0.1 mmol of β-Ala-Wang resin (0.38 mequiv. of amino group/g) using an automatic peptide synthesizer (Model 433A, Applied Biosystems Inc.). Trifunctional amino acids were protected on their side-chain as follows: trityl (Trt) for Gln; t-butyl (t-Bu) for Ser and Tyr, Fmoc for Lys

and pentamethylchroman (Pmc) for Arg. The purity of peptides was verified by: (i) analytical reversed-phase HPLC (ii) amino acid analysis after acid hydrolysis (6 N HCl/1% phenol (mass/vol.), 20 h, 120°C, N2 atmosphere), and (iii) mass determination by matrix-assisted laser desorption ionization-time of flight (MALDI-TOF) mass spectrometry.

HIV-$1_{NL\ 4-3}$ infection of C8166 cells

Samples of 3 x 10^5 C8166 cells were placed in 96-well plates in a volume of 100 µl of culture medium containing various concentrations of peptide. After a 1 h treatment at 37°C, 100 µl of viral solution of HIV-$1_{NL\ 4-3}$ were added. The cells were exposed to the virus for 1 h at 37°C at a multiplicity of infection of 1,000 $TCID_{50}$ per ml. After thorough washing, cells were replaced in 1 ml of R10 with the treatment in 24-well plates and cultured in a 37°C incubator. C8166 culture medium was replaced at Day-4 post-infection. During this assay, treatment with peptide was permanent (before, during and after infection). Assays on C8166 cells have been performed at least twice and in duplicate. Toxicity was evaluated by daily cell count and trypan-blue exclusion assay. Infection of C8166 T-cells with HIV-$1_{NL\ 4-3}$ was assessed by virus-induced cytopathic effects (syncytia formation) and by quantification of p24 viral protein in the culture supernatants. Measurements of HIV-1 $p24^{gag}$ concentrations in the culture supernatants were achieved by ELISA, with a detection cut-off of 5 pg/ml (p24 HIV kit, NEN Dupont, Belgium; Quanti-Kine software, RILAB, Genova, Italia).

Infection of human peripheral blood lymphocytes

Samples of 10^6 human PBLs were placed in 96-well plates in 100 µl of R10 containing various concentrations of peptide. After 1 h treatment at 37°C, 100 µl of viral solution of HIV-1_{NL-4-3} were added. The cells were exposed to the virus for 1 h at 37°C at a multiplicity of infection of 100 $TCID_{50}$ per ml. After thorough washing,

cells were replaced in 1 ml of culture medium in 24-well plates and cultured in a 37°C incubator flushed with 5% CO2. The PBL culture medium was replaced every 3-4 days. The cell viability was assessed by cell counts and trypan-blue exclusion assay. The viral production in the culture supernatant was quantified by p24 ELISA test, as described earlier. All the experiments have been done in blind-tests. Tests have been achieved in duplicate.

Results

Inhibition of HIV-$1_{NL\ 4-3}$ infection of PBMCs and C8166 cells by $[RQGYS]_2$-K-βAla

A). $[RQGYS]_2$-K-βAla peptide (with five residues) was able to inhibit syncytium formation and p24 production of HIV-$1_{NL\ 4-3}$ infected C8166 cells. 100% of inhibition was obtained at a concentration of 1μM at low concentration.

B). $[RQGYS]_2$-K-βAla (at a concentration of 0.1 μM) induced a decrease (100%) in the p24 concentration in the culture supernatants from infected human PBLs.

TABLE

Inhibition of HIV-1 infection of C8166 cells by MBPCS

Peptide	P24 (pg/ml)		Syncytium formation at peptide concn of:	
	1μM	10 μM	1μM	10 μM
1. $[RQGYSPL]_2$-K-βA	0	0	-	-
2. $[RQGYSP]_2$-K-βA			+	-
3. $[RQGYS]_2$-K-βA	0	0	-	-
4. $[RQGY]_2$-K-βA			±	±
5. $[RQG]_2$-K-βA			±	-
6. AZT	0	0	-	-
7. No peptide	25000	23000	++	++

Symbols:

++ number of syncytia present in the well was similar to that in control untreated wells (35 to 40 syncytia per well),

+ presence of 4 to 34 syncytia in the well

± presence of 1 to 3 syncytia in the well

\- total absence of syncytia in the well

C). $[RQGY]_8$-$[K]_4$-$[K]_2$-K-βA was able to inhibit syncytium formation and p24 production of HIV-$1_{NL\ 4-3}$ infected C8166 cells. 100% of inhibition was obtained at a concentration of 5 μM.

The multibranched peptides were not toxic for cells, even at a concentration of 50 μM (data not shown).

CLAIMS

1. A multiple branch peptide construction comprising a core matrix to which are bonded from 2 to 16 peptides, each of which has amino acid sequence RQGYS.

2. A multiple branch peptide construction comprising a core matrix to which are bonded from 8 to 16 peptides, each of which has the amino acid sequence RQGY.

3. A peptide construction according to claim 1 or claim 2 in which the core matrix is comprised of lysine residues.

4. A peptide construction according to any preceding claim in which there are spacers between the core matrix and the peptides.

5. A peptide construction according to claim 1 in which the peptides are peptide analogues.

6. A peptide construction according to claim 1 in which the peptides include at least one D-amino acid residue.

7. A peptide construction according to any preceding claim which is non-immunogenic at a blood concentration of up to 10^{-4} molar.

8. Liposomes having a sufficient size for white blood cell internalisation, the liposomes containing a multiple branch peptide construction according to any preceding claim.

9. Liposomes according to claim 8 which have an average size of greater than 150 nm.

10. Liposomes according to claim 8 which have an average size of approximately 250 nm to 400 nm.

11. Liposomes according to any of claims 8 to 10 which contain above 8% by weight of the multiple branch peptide construction.

12. A medicament comprising a multiple branch peptide construction according to any of claims 1 to 7 or liposomes according to any of claims 8 to 11 in admixture with a pharmaceutically acceptable diluent or carrier.

13. A method for the therapeutic treatment of patients with HIV infections, the method comprising administering to the patient a multiple branch peptide construction according to any of claims 1 to 7, liposomes according to any of claims 8 to 11 or a medicament according to claim 12.

14. A method according to claim 13 in which the multiple branch peptide construction, the liposomes or the medicament is administered to the patient in an amount sufficient to induce in the patient a blood concentration of less than 10^{-4} molar.

15. A method according to claim 13 or claim 14 in which the multiple branch peptide construction, the liposomes or the medicament is administered to the patient in an amount sufficient to induce in the patient a blood concentration of more than 10^{-7} molar.

INTERNATIONAL SEARCH REPORT

International Application No
PCT/EP 03/04353

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 C07K14/16 A61K38/16 A61K9/127

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 7 C07K A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)
CHEM ABS Data, EPO-Internal, MEDLINE

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 98 29443 A (MABROUK KAMEL ;ROCHAT HERVE (FR); RIETSCHOTEN JURPHAAS VAN (FR); S) 9 July 1998 (1998-07-09) cited in the application	1-7, 12-15
Y	the whole document	8-11

X	SABATIER J M ET AL: "Anti-HIV activity of multibranched peptide constructs derived either from the cleavage sequence or from the transmembrane domain (gp41) of the human immunodeficiency virus type 1 envelope." VIROLOGY. UNITED STATES 15 SEP 1996, vol. 223, no. 2, 15 September 1996 (1996-09-15), pages 406-408, XP002253691 ISSN: 0042-6822	1-7, 12-15
Y	the whole document	8-11

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[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

° Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
8 September 2003	06/10/2003

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL – 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Mundel, C

Form PCT/ISA/210 (second sheet) (July 1992)

INTERNATIONAL SEARCH REPORT

International Application No
PCT/EP 03/04353

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication,where appropriate, of the relevant passages	Relevant to claim No.
X Y	WO 99 34777 A (CELLPEP S A ;ROUGE BONABES OLIVIER DE (FR)) 15 July 1999 (1999-07-15) the whole document	1-7, 12-15 8-11
Y	YAHI N ET AL: "MULTIBRANCHED V3 PEPTIDES INHIBIT HUMAN IMMUNODEFICIENCY VIRUS INFECTION IN HUMAN LYMPHOCYTES AND MACROPHAGES" JOURNAL OF VIROLOGY, THE AMERICAN SOCIETY FOR MICROBIOLOGY, US, vol. 68, no. 9, 1 September 1994 (1994-09-01), pages 5714-5720, XP002063190 ISSN: 0022-538X the whole document	1-15
Y	US 5 622 933 A (FENOUILLET EMMANUEL ET AL) 22 April 1997 (1997-04-22) the whole document	1-15
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INTERNATIONAL SEARCH REPORT

International application No.
PCT/EP 03/04353

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. [X] Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

Although claims 13-15 are directed to a method of treatment of the human/animal body, the search has been carried out and based on the alleged effects of the compound/composition.

2. [ ] Claims Nos.:
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:

3. [ ] Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II *Observations where unity of invention is lacking (Continuation of item 2 of first sheet)*

This International Searching Authority found multiple inventions in this international application, as follows:

1. [ ] As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.

2. [ ] As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. [ ] As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:

4. [ ] No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

[ ] The additional search fees were accompanied by the applicant's protest.

[ ] No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No
PCT/EP 03/04353

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